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Histopathological and biological studies of the effect of cadmium on *Rhinella arenarum* gonads

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ABSTRACT

This study was to determine the lethal dose 50 (LD_{50}) of $CdCl_2$ in adult *Rhinella arenarum* and analyzed the effect of two sublethal doses (0.5 and 5 mg/kg) of the xenobiotic in gonads. The 48 h LD_{50} were 50.0 and 49.8 mg/kg for males and females respectively. Alterations in the ovary were evidenced by nuclear pleomorphism and cytoplasmic vacuolization of the oocytes at the early stages of development with the highest dose and an increase in the population of atretic oocytes. In the interstitial tissue we noticed congestion, edema and fibroblast proliferation. The nuclear maturation of the oocytes was affected by the xenobiotic in a dose- and time-dependent manner. In males, treatment with 5 mg/kg of cadmium (Cd) caused a decrease in the concentration, viability and straight progressive motility of sperm while there was an increase in immotile sperm. Testis histopathology revealed dilated seminiferous tubules, disappearance of cysts, tissue disorganization and leukocyte infiltration. Numerous germ cells showed hydropic tumefaction or signs of focal necrosis. The Cd content in animals intoxicated gonads with the highest sublethal dose was significantly higher than in the control. Results indicate that *R. arenarum* gonads are target for the xenobiotic, compromising the formation of gametes competent for fertilization, the effective $CdCl_2$ dose being 5 mg/kg.

1. Introduction

The decrease in the diversity of amphibian species in their natural habitats has been documented for the last years in the six continents (Bishop et al., 1999; Carey and Bryant, 1995; Kucken et al., 1994; Sparling et al., 2000). Considering that the quality of the land and aquatic environments can ensure the persistence of amphibian populations (Kucken et al., 1994), chemical pollution and the physical alteration of the environment are the main causes proposed to explain the decline in the different species worldwide (Bishop et al., 1999; Collins and Storfer, 2003). It is known that various anthropogenic activities cause a great variety of contaminants, one of them being cadmium (Cd). This heavy metal is of great interest due to its physico-chemical properties. That is why it is frequently used in industry, *e.g.*, in the manufacture of cell phone batteries (a mass consumption product), and also for the manufacture of photovoltaic cells destined to the solar panels proposed nowadays as an alternative to fossil fuels (Bhattacharyya et al., 2000; Järup et al., 1998). It should be noted that these products have a finite life and that, if not recycled safely, they can contribute to the worldwide problem of toxic wastes (Fowler, 2009). Cd is extremely toxic because it cannot undergo biodegradation or biotransformation and has a prolonged biological half-life (15–30 years) (Henson and Anderson, 2000) and a low excretion rate. This explains why it is a xenobiotic that accumulates mainly in liver and kidneys, where it may reach concentrations higher than the ones allowed by the Occupational Safety and Health Administration (OSHA, 1992). In lesser amounts, it accumulates in erythrocytes, lungs, pancreas, thyroid, testis, salivary glands and placenta (Bhattacharyya et al., 2000; Henson and Anderson, 2000).

In amphibians, most ecotoxicological and toxicological studies have focused mainly on aquatic pollution and on the early stages of their life cycle, demonstrating that Cd has adverse effects on survival, growth and behavior (Gross et al., 2007; James and Little, 2003). It has also been postulated that embryos and larvae are more sensitive than young and adult specimens (Herkovits et al., 1997). However, it is important to note that the viability of the population depends on the maintenance of the physiological conditions at all stages of the life cycle, so that further studies are required in adults, whose survival has a high impact on population because of their reproductive capacity. Among amphibians, *Xenopus laevis*, a species native to South Africa and totally aquatic, has been used as a model

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for toxicity studies by Cd at the laboratory level (Fort et al., 2001, 2002; Herkovits et al., 1997; Lienesch et al., 2000; Mouchet et al., 2007).

It is important to point out that the evaluations carried out have demonstrated that there may be differences in interspecific sensitivity (Birge et al., 2000). Consequently, it should not be assumed that the effects of Cd and their magnitude are common to all species. Therefore, the need arises to analyze the effect of a contaminant on specimens collected from their natural habitat and at the regional level.

In our region (Argentine northwest), pollution is a difficult problem to solve due to the existence of different factors: misuse of pesticides, lack of adequate treatment of industrial (citrus industry, paper industry, among others) and urban effluents, lack of complying with existing dangerous residues laws (Sosa et al., 1999), and the presence of a mineral duct through the region. Studies conducted by national institutions in charge of monitoring soil and water pollutants indicate that regional dams, their affluents and the adjacent soil contain heavy metals above normal levels. The materials found are Cd, arsenic, lead, mercury, zinc, copper and silver, among others. With respect to Cd, a level higher than 1 mg/L has been reported in the waterways (Comisión Nacional de Energía Atómica (CNEA), informe N° 260537) inhabited by *R. arenarum* specimens.

It is known that the presence of heavy metals and complex organic substances in the soil and water has a great impact on the ecosystem and on public health in general (Thomann, 1982). At present there are no data concerning the negative influence of these pollutants on regional species.

Rhinella arenarum, an anuran species native to the Argentine northwest, is a useful tool for the evaluation and standardization of bioassays because of its sensibility, ease of handling at the lab level and the reproducibility and reliability of its morphological and physiological responses under controlled conditions (Ferrari et al., 2005). Its life cycle takes place in both aquatic and land environments. The embryonic and larval stages take place in fresh water while once metamorphosis is completed the animals live in a land habitat and the adults return to the water to breed and lay their eggs. The characteristics of these breeding sites may have important effects on fertilization, metamorphosis and survival.

Reproductive impairment by Cd has been well reported for sea urchin (Au et al., 2000, 2001a,b), fish (Dietrich et al., 2010), ovine (Leoni et al., 2002) and rat (El-Demerdash et al., 2004; Yang et al., 2006), while in amphibians there are few studies on the subject. At the level of spermatogenesis in *Rana hexadactyla Lesson* (Kasinathan et al., 1987) and *Bufo melanostictus* (Biswas et al., 1976), it has been demonstrated that Cd causes a decrease in secondary spermatogonial and primary spermatocytic stages in the seminiferous tubules. It has also been reported that Cd interferes with oocyte development in *X. laevis* (Fort et al., 2001, 2002; Lienesch et al., 2000). Since there are few studies on the quality of amphibian gametes treated with sublethal doses of Cd in the laboratory, in this investigation we evaluated the reproductive status of adult *R. arenarum* after acute Cd intoxication. Ovary pathology, oocyte growth and maturation (germinal vesicle breakdown) were evaluated in the females, and testis pathology, sperm count, viability and motility in the males. In order to choose the sublethal doses, we previously determined the lethal dose 50 (LD_{50}) of the xenobiotic.

The results of this study will enable a better understanding of the acute effects of Cd pollution on the reproductive success of *R. arenarum* as well as the determination of measurable physiological parameters to be used in bioassays. It is important to notice that bioassays are mandatory in the USA, Canada, Japan and EU Member States, where they are implemented through their respective Environmental Protection Agencies, while in our country, despite their importance, they are seldom used.

2. Materials and methods

2.1. Animals

Sexually mature *R. arenarum* males and females (100–150 g body weight) were collected during the reproductive period (September–November) in the neighborhood of San Miguel de Tucumán, Argentina.

2.2. Preparation of the cadmium solution

Cadmium chloride ($CdCl_2$) (Sigma–Aldrich of Argentina CAS No. 10108-64-2, 99.0% pure) was dissolved in distilled water.

2.3. Lethal dose study

In order to establish the LD_{50}, we assayed the effect of the following dose of $CdCl_2$: 0.5, 5, 50, 75 or 100 mg/kg injected into the dorsal lymphatic sac at a volume of 0.9–1 mL in distilled water. The number of animals used was five for each dose run in quadruplicate ($n = 20$). Using a probit dose–mortality curve based on mortality at 48 h, we determined the lethal dose of $CdCl_2$ with the SigmaPlot 11 software (Standard curves analysis—Pharmacology section).

The model fit (nonlinear regression) was:

$$f = \hat{a} + \frac{(\hat{b} - \hat{a})}{1 + \left| x/\hat{\mathrm{LD}}_{50} \right|^{-\hat{c}}}$$

where f = response (dependent variable); x = dose (independent variable); $\hat{a}$ = minimum mortality rate; $\hat{b}$ = maximum mortality rate; $\hat{c}$ = hillslope and $\hat{\mathrm{LD}}_{50}$ = lethal dose 50.

2.4. Treatment with cadmium

R. arenarum males and females were injected into the dorsal lymphatic sac daily for 15 days with either 0.5 or 5 mg/kg of $CdCl_2$, which were chosen as the sublethal doses in this study. Control animals were injected by the same route with 0.9–1 mL distilled water during the same period. Although it is well-known that the natural routes of assimilation of environmental components are skin and foods, we chose to inject the xenobiotic into the dorsal lymphatic sac to ensure the total incorporation of the doses assayed (Lienesch et al., 2000) and thus evaluate their effect. The doses of $CdCl_2$ used in the present study were selected considering: (a) the doses that had an effect on a parameter of the reproductive activity of *R. arenarum* without causing mortality in the specimens; (b) the doses used by other investigators: 0.5–5 mg/kg body weight injected into the dorsal lymph sac of *X. laevis* every 2 days for 21 days (Lienesch et al., 2000); 0.5 mg/kg in a single subcutaneous injection in *R. hexadactyla Lesson*, which was sacrificed at 3 and 7 days (Kasinathan et al., 1987); and 0.5 mg/kg in a single subcutaneous injection into *B. melanostictus* (Biswas et al., 1976), which was sacrificed at 3 and 7 days. Preliminary studies in our laboratory showed that the treatment with a single daily dose of 5 mg/kg for 15 and 20 days did not cause mortality in the specimens and that the mean Cd concentration in the liver was 263 and 390 μg/g dry weight respectively. In *Rana ridibunda*, Loumbourdis and Vogiatzis (2002) determined an average Cd concentration of 857.62 μg/g in the liver after treatment with 200 ppm $CdCl_2$ for 30 days. The average Cd concentration in the liver determined by these authors is much higher than the one found in our assays, although they found no mortality of the specimens.

During the treatment period, treated animals and controls were kept in boxes with appropriate humidity at room temperature. Animal maintenance and experimental procedures were in accordance

with the "Guide for Care and Use of Laboratory Animals" (European Communities Council and Directive, 1986).

2.5. Histological evaluation of gonads

At the end of the treatment testes samples were fixed in formol 10%, while the ovary pieces were fixed in Ancel and Vintemberger. Then all samples were dehydrated and included in paraffin. The slices (6 μm thick) were stained with hematoxylin–eosin for routine diagnostics or Masson's trichrome to detect fibrous tissue (collagen).

Ovarian follicles were categorized as described by Valdez Toledo and Pisanó (1980).

Each assay included 3 control and 3 treated animals per dose.

2.6. Oocyte maturation

Ovaries of control (n = 3 animals) and treated (n = 3 animals per dose) females were removed at the end of the treatment period. Fully grown oocytes with a diameter of 1.5–1.7 mm and surrounded by one layer of follicle cells were isolated from the both ovaries with watchmaker's forceps under a stereoscopic microscope and randomly grouped in lots of about 150 follicles. Each lot was incubated in Ringer's solution in the presence of different progesterone doses (0.5, 1 or 2 μg/mL) for different time periods (4, 8, 12 or 24 h). The controls were incubated under the same conditions but without the addition of progesterone. The presence of the germinal vesicle was examined under the stereoscopic microscope.

2.7. Identification of apoptotic cells

Control (n = 3 animals) and treated testes (n = 3 animals per dose) were studied.

The presence of apoptotic cells in formalin-fixed paraffin-embedded testes sections were analyzed by terminal deoxynucleotide transferase mediated dUTP nick end-labeling (TUNEL) using Roche kit (Cat. No. 11 684 817 910) with immunoperoxidase visualization according to the manufacturer's protocol. The TUNEL positive cells appeared brown.

2.8. Obtainment of sperm suspension and evaluation of sperm quality parameters

At the end of the treatment the testes of treated animals (n = 5 males per dose) and controls (n = 5 males) were removed and dilacerated by mincing testes in Ringer's solution pH 7.4 with or without 10 mM Tris–HCl pH 7.4 to obtain a sperm suspension. Suspensions were filtered through a 30 μm mesh nylon screen to remove large pieces of testis debris. Aliquots of the filtrate were used to evaluate: (A) sperm concentration (cell/mL) determined by means of a Neubauer chamber, (B) percentage of live spermatozoa by eosin staining and (C) percentage of sperm motility observed with a high power microscope (×400). They were classified into immotile sperm, with *in situ* motility and with straight progressive motility. To analyze B and C we worked with 50 μL of a suspension of 1×10^7 cell/mL.

2.9. Determination of cadmium concentration

To determine Cd levels, we weighed between 0.02 and 0.2 g of tissue from the testis and ovary of control animals (n = 3) and animals treated (n = 3) with $CdCl_2$ 5 mg/kg.

Then an acid digestion was performed with concentrated nitric acid, 30% hydrogen peroxide and concentrated sulfuric acid (10:5:1 ratio) in Teflon containers decontaminated and brought to volume with distilled water. The Cd determination was carried out with an Atomic Absorption Spectrometer (PerkinElmer) (Notwalk, CT, USA) AAnalyst 100 equipped with a Graphite Furnace, HGA 800 and an AS-72 autosampler. We used a Cd hollow cathode lamp as the radiation source with 10 mA lamp current. Pyrolytically coated tubes with integrated platforms were used (Part. No. B3000407, PerkinElmer). The graphite furnace program followed was the default program provided by the software manufacturer. Peak area measurements were made at 228.8 nm peak in triplicate. Quantification was performed using a calibration curve determined automatically by the autosampler with Cd standard solutions prepared from the 10 μg/mL Cd stock solution. The volume injected was 20 μL for all cases: calibration blank (5%, v/v nitric acid), calibration standards and sample solutions (in some cases, the samples were diluted). Modifier working solutions of magnesium nitrate and palladium nitrate were used. The detection limit was 0.09 μg/mL and the quantification limit was 0.2 μg/mL. The accuracy of the method was ±10% and recovery of 90–110%. Values were expressed as μg/g wet weight.

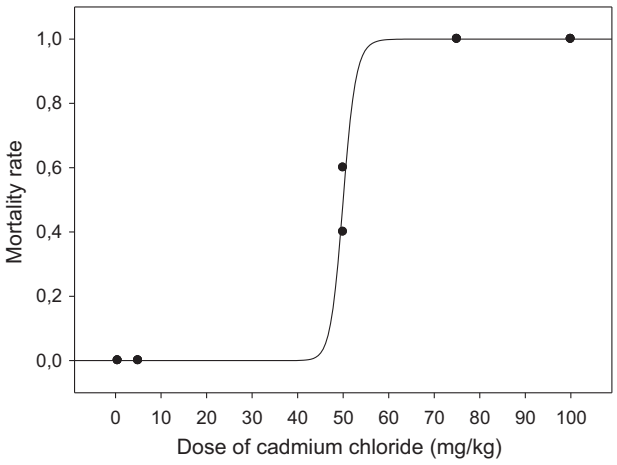

Fig. 1. Dose–mortality curve 48 h after a single subcutaneous injection of $CdCl_2$ into the dorsal lymph sac of *R. arenarum* males. The curve was based on probit regression analysis. (n = 20 animals per dose).

2.10. Statistical analysis

Statistical analysis was performed using the non-parametric Kruskal–Wallis test followed by the multiple comparison method (Dunn's Method). All statistical comparisons were made above the 95% confidence level.

3. Results

3.1. Lethal dose

According to the dosage–mortality curve at 48 h, the LD_{50} for $CdCl_2$ was determined as 50.0 and 49.8 mg/kg for males (Fig. 1) and females respectively.

Using the model fit (nonlinear regression) expressed in materials and methods we have $\hat{a} = 0$; $\hat{b} = 1$; $\hat{LD}_{50} = 50$; $\hat{c} = 37.9$ with R^2 = 0.9936 for the males and $\hat{a} = 0$; $\hat{b} = 1$; $\hat{LD}_{50} = 49.8$; $\hat{c} = 50.8$ with R^2 = 0.9952 for the females.

3.2. Effect of cadmium on the ovary

Analysis the ovarian lobes from control (Fig. 2A) and treated animals with 0.5 mg/kg of Cd (Fig. 2B) showed preserved histological characteristics in which we can identify oocytes at different developmental stages as well as interstitial tissue with a normal appearance (Fig. 2C). The histoarchitecture of the ovary of

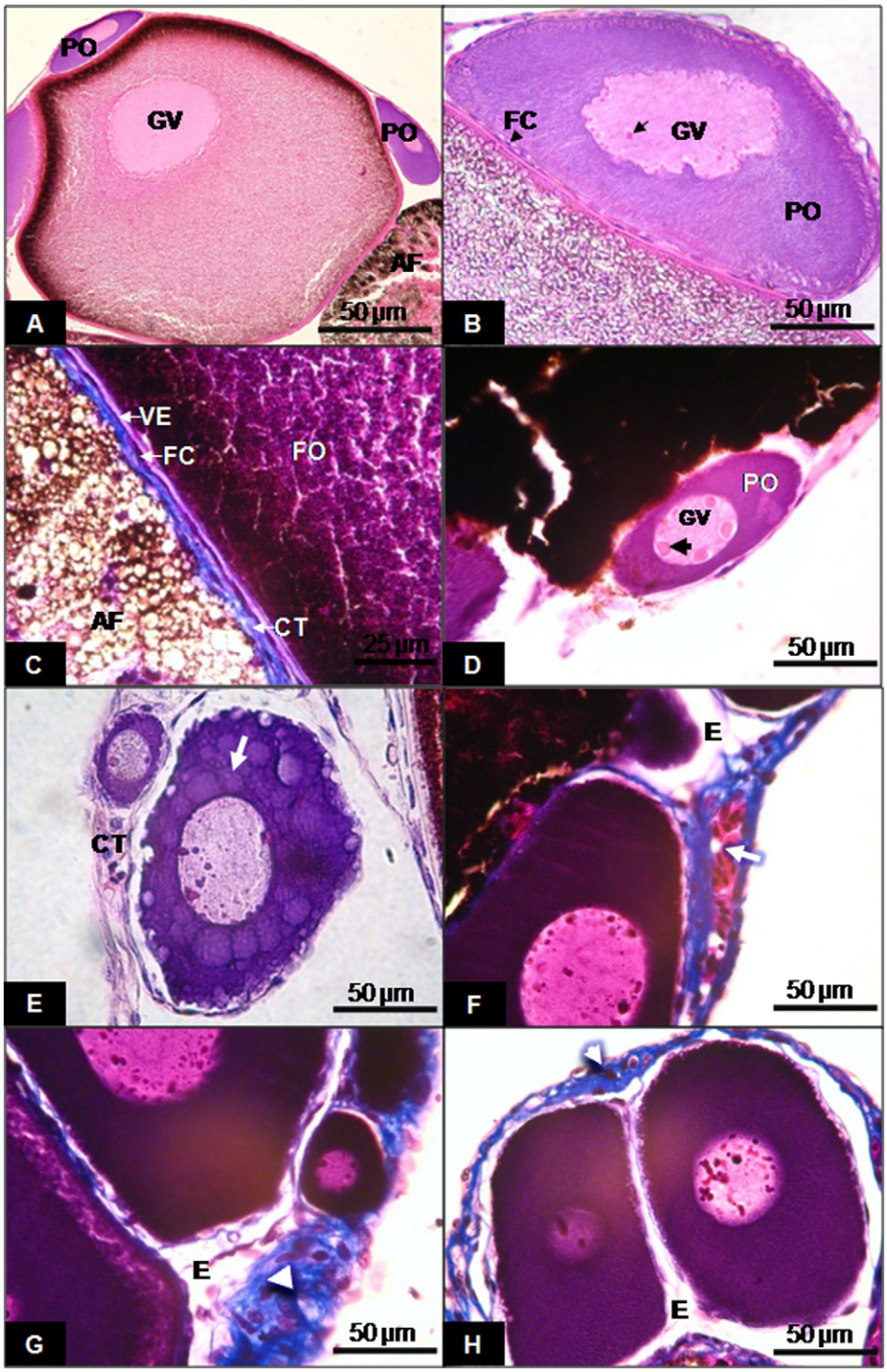

Fig. 2. Light micrographs of ovarian tissue of *R. arenarum* after treatment with Cd. (A) Controls. No obvious alterations were observed in fully grown and primary vitellogenic oocytes (PO). Germinal vesicle, GV; atretic follicle, AF. H&E stain 40×. (B) Treated with 0.5 mg/kg. Normal primary vitellogenic oocytes (PO) showing a germinal vesicle (GV) in a central position. Nucleoli (arrow); nucleus of follicular cell, FC. H&E stains 630×. (C) Controls. Fully grown oocyte (FO) and atretic follicle (AF). Follicular cell, FC; vitelline envelope, VE; connective tissue, CT. Masson's trichrome stain 630×. (D) Treated with 5 mg/kg. Germinal vesicle (GV) of primary vitellogenic oocytes (PO). Note variations in shape, size and number of nucleoli (arrow). H&E stain 630×. (E) Treated with 5 mg/kg. Oocytes at the early developmental staces with cytoplasmic vacuolization (arrow). Connective tissue, CT. H&E stain 630×. (F) Treated with 5 mg/kg. Alteration of the interstitial tissue with dilation of vascular lumens (arrow) and edema (E). Masson's trichrome stain 630×. (G) Treated with 5 mg/kg. Alteration of the interstitial tissue with nodular fibroblastic proliferation (arrow) and edema (E). Masson's trichrome stain 630×. (H) Treated with 5 mg/kg. Alteration of the interstitial tissue with fibroblastic proliferations in strips (arrow) and edema (E). Masson's trichrome stain.

animals treated with the highest sublethal Cd dose (5 mg/kg) showed serious changes compared with the control. The most affected germ cells were follicular oocytes at the earlier developmental stages (previtellogenic oocytes). In fact, they showed nuclear and cytoplasmic alterations such as variations in the shape, size and number of nucleoli (Fig. 2D) and cytoplasmic vacuolization. This last process started with small vacuoles of different sizes and shapes of peripheral distribution that progressed toward the

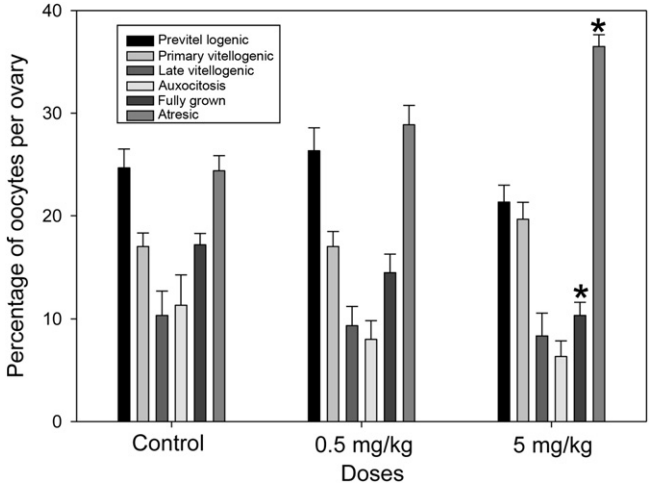

Fig. 3. Effect of cadmium on percentage of oocytes at different developmental growth and on percentage of atretic follicles per ovary. Values are expressed as percentage of oocytes at different stages of maturation and atretic follicles from ovaries of different animals: mean ± SE ($n = 9$ animals per dose). (*) indicates significant difference with respect to control ($p < 0.05$).

central region associated with hydropic tumefaction (Fig. 2E). At the interstitium, between the follicles, we observed dilated capillary lumens (congestion), edema (Fig. 2F), fibroblast proliferation in nodules (Fig. 2G) or strips (Fig. 2H) and cumulus of cells with hyperchromatic to pyknotic nuclei possibly related to focal necrosis. It is important to note that the histological damage is not homogeneous but circumscribed to certain areas of the interstitium.

Cd affects oocyte growth. In fact, the percentage of fully grown oocytes showed a significant decrease ($p < 0.05$) in animals treated with 5 mg/kg of Cd compared with controls. At the same dose a significant increase ($p < 0.05$) was observed in the percentage of atretic follicles compared with controls. The above effects were not detected at the lower Cd doses (0.5 mg/kg) assayed (Fig. 3).

3.3. Effect of cadmium on oocyte maturation

The effect of Cd on oocyte nuclear maturation, expressed as percentage of germinal vesicle breakdown (% GVBD), was analyzed using progesterone as an inducer of the process. Our results indicated that Cd affected nuclear maturation in a time- and dose-dependent manner (Fig. 4A–D). When full grown follicle oocytes from females treated with the 5 mg/kg Cd dose were incubated for short periods with 1 μg/mL progesterone, a low GVBD percentage was observed. The inhibition caused by Cd was 95% and 73% at 4 and 8 h respectively. A smaller inhibition of % GVBV was found with animals treated with the 0.5 mg/kg dose in all progesterone incubation periods studied.

3.4. Effect of cadmium on testis

Testes from control animal (Fig. 5A) and animals treated with 0.5 mg/kg of Cd (Fig. 5B) showed preserved cytoarchitecture throughout the study period. Also, all stages of germ cells (spermatogonia, spermatocytes, spermatids and sperm) coexisted in the seminiferous tubules. The interstitial tissue between the seminiferous tubules was intact, with no cellular infiltration or inflammatory processes (Fig. 5B). Germ cells and Sertoli cells did not show significant alterations (Fig. 5B). In contrast, testes from animals treated with the 5 mg/kg Cd dose showed important alterations such as dilated seminiferous tubules with disappearance of cysts, tissue disorganization (Fig. 5C) and leukocyte infiltration. Numerous germ cells also showed hydropic tumefaction or signs of focal necrosis (Fig. 5D). Others showed fragmented chromatin leading to small nodular masses that appeared as basophilic apoptotic bodies and condensed in the nucleus periphery. These cells were usually separated from neighboring cells and became surrounded by a clear space or pseudo-halo (Fig. 5D). This observation was confirmed with the TUNEL-positive cells (Fig. 5E). In the interstitial tissue, enlargement of the vascular lumen was observed (Fig. 5F). It should be noted that in all examined sections the tubules showed similar alterations. However, normal tubules or tubules with mild degeneration were observed in the testes samples but these were only a small minority.

3.5. Effect of cadmium on sperm quality

When analyzing sperm quality (sperm concentration, sperm viability and type of sperm motility), we observed important differences between control males and males treated with the different sublethal Cd doses assayed (Fig. 6A–C). Thus, in animals treated with 5 mg/kg Cd we observed that concentration, viability and straight progressive motility of sperm were significantly lower ($p < 0.05$) than in the control. In addition, a significant increase ($p < 0.05$) in immotile sperm was detected. In contrast, the analysis of concentration and viability in animals treated with 0.5 mg/kg Cd did not show significant differences with respect to the control, although the percentage of immotile sperm and sperm with *in situ* motility in animals treated with this dose was significantly higher than in control ($p < 0.05$).

3.6. Cadmium content in ovary and testis

Taking into account the effects observed in ovary and testis after Cd intoxication, we determined by ET AAS the concentration of Cd in those organs. Significant differences ($p < 0.05$) were found in Cd concentrations between the gonads of control animals and those of animals treated with the 5.0 mg/kg Cd dose. The values corresponding to ovarian tissue were 0.049 ± 0.005 μg/g ($n = 3$) and 90.5 ± 4.618 μg/g ($n = 3$) respectively and for testicular tissue they were 0.686 ± 0.028 μg/g ($n = 3$) and 216.0 ± 9.237 μg/g ($n = 3$) respectively.

4. Discussion

The growing concern about the decline of amphibian populations emphasizes the need to widen our knowledge of the impact of heavy metal pollution on adult organisms. Cd could cause a decrease in those populations due to its lethal or sublethal effects. In order to make a good choice of the different sublethal doses and in view of the lack of data on the lethal dose in adult specimens of the amphibian *R. arenarum*, in the present work we determined the DL_{50} of $CdCl_2$ in males and females. The values of DL_{50} found did not show significant differences between the sexes. On the basis of these results, we selected the doses of 0.5 and 5 mg/kg of Cd for the study of the effect of sublethal doses. For Cd administration we chose the subcutaneous injection (dorsal lymphatic sac) as an intoxication way, since the oral route is neither reliable nor is it easy to ensure the amount that the animal receives, although this last way is one of the natural forms of exposure.

The survival of a species depends on its reproductive capacity. In this work we analyzed the effect of Cd on oocyte nuclear maturation, an important event in the reproduction taking into account that only mature gametes are able to ovulate and be fertilized. In *R. arenarum*, as in other amphibian species, nuclear maturation is physiologically induced by progesterone (Romero et al., 1998). After the steroid interaction with its membrane receptor, a decrease in intracellular cAMP, changes in Ca^{2+} fluxes and an increase in the

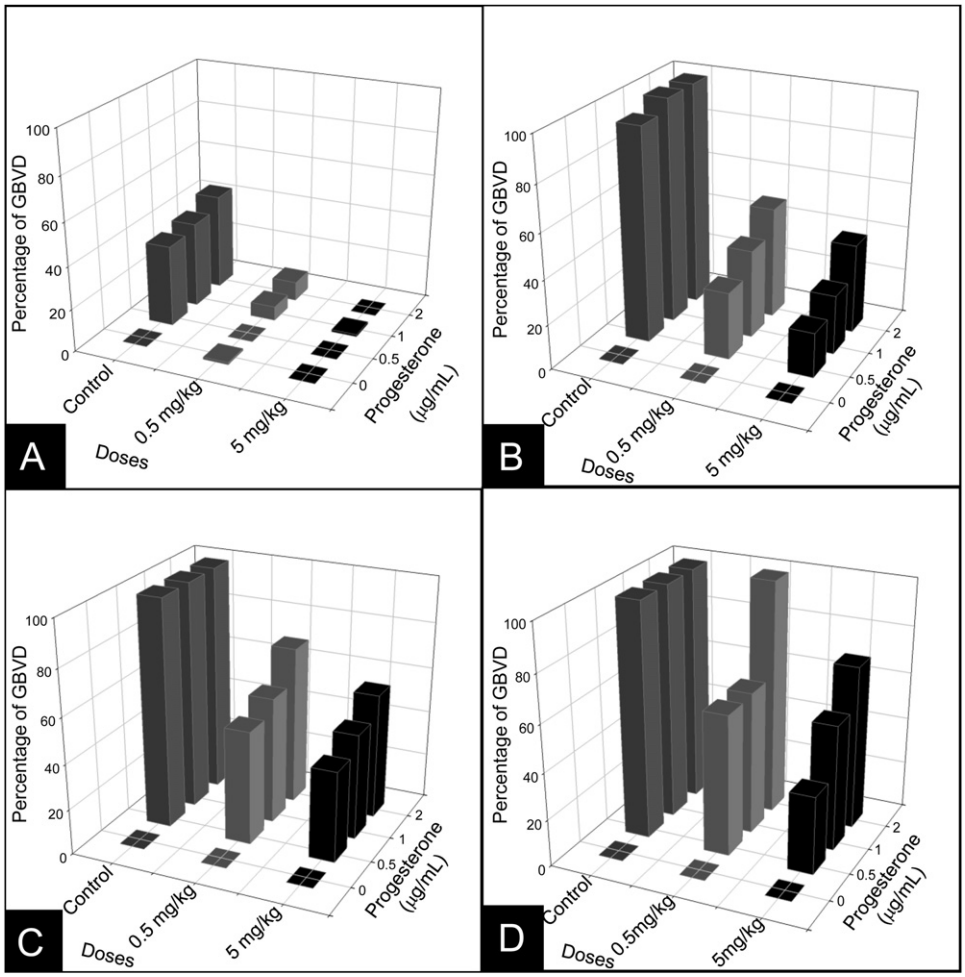

Fig. 4. Effect of different cadmium doses on progesterone-induced germinal vesicle breakdown (GVBD) at 4 h (A), 8 h (B), 12 h (C) and 24 h (D). Data are reported as mean ± SE ($n = 3$ animals per dose). (*) indicates significant difference with respect to control ($p < 0.05$).

maturation promoting factor (MPF) occur (Baulieu and Schorderet-Slatkine, 1983; Romero et al., 1998). Our results in *R. arenarum* demonstrated that Cd exerts an inhibitory effect on nuclear maturation, expressed as a decline in GVBD percentage, this inhibition being dose- and time-dependent. Thus, a high inhibitory effect was observed at the 5 mg/kg Cd dose and during short incubation periods. Lowest inhibition was found at the dose of 0.5 mg/kg. The inhibition of oocyte nuclear maturation by the xenobiotic could be due to a block to MPF formation, a process that takes place around 4–6 h after the onset of progesterone action (Baulieu and Schorderet-Slatkine, 1983). Another mechanism that could explain this inhibition is that Cd can alter the intracellular concentration of Ca^{2+} blocking the release of the stored Ca^{2+} by the inhibition of IP_3 activity (Martelli et al., 2006).

The histopathological analysis of the ovary of *R. arenarum* exposed to sublethal doses of Cd revealed significant changes not only in the oocytes but also in the ovary interstitial tissue. Thus, when comparing the control ovaries with those exposed to the highest dose of Cd, we observed that in both groups oocyte growth continued, and we could identify oocytes at different stages of development as well as the presence of atretic follicles. However, the number of oocytes, especially fully grown ones, was lower and at the same time there was a greater susceptibility to Cd in oocytes at the first stages of development, which showed oocyte degeneration. The decrease in the growth of oocytes would be explained by the toxic effect of Cd at the doses assayed on *R. arenarum* hepatic cells (unpublished results). These cells synthesize and secrete vitellogenin, a yolk precursor, whose accumulation is a determining factor in oocyte growth (Skipper and Hamilton, 1977). In addition, it has been reported that Cd inhibits vitellogenesis in fish due to its antagonistic effect on the estrogen receptor, a hormone that stimulates yolk formation (Guével et al., 2000). Jeong et al. (2000) demonstrated a time- and dose-dependent Cd-induced inhibition of gap junction intercellular communication caused by decreased expression of connexins. This effect would limit the metabolic activity of the oocytes, inhibiting oocyte growth. Our findings are consistent with those of Lienesch et al. (2000) in which *X. laevis* specimens were exposed to $CdCl_2$ (0.5, 3 or 5 mg/kg) for 21 days. These authors found that Cd caused a significant decrease in the oocyte population at phases IV and VI at all Cd concentrations assayed. However, with the methodology used, they were unable to determine whether oocytes at stage II underwent degeneration. In the present study we determined the presence of a significant increase in the population of atretic oocytes in females exposed to the highest doses.

It is important to identify which gonad component is affected by the xenobiotic in order to predict Cd impact on reproduction. In our study, we determined that Cd damages previtellogenic oocytes, which would lead to temporary infertility since oogonia did not show alterations (results not shown). One of the most probable mechanisms of cell damage is pronounced oxidative stress. The enzyme systems that eliminate free radicals and their derivatives

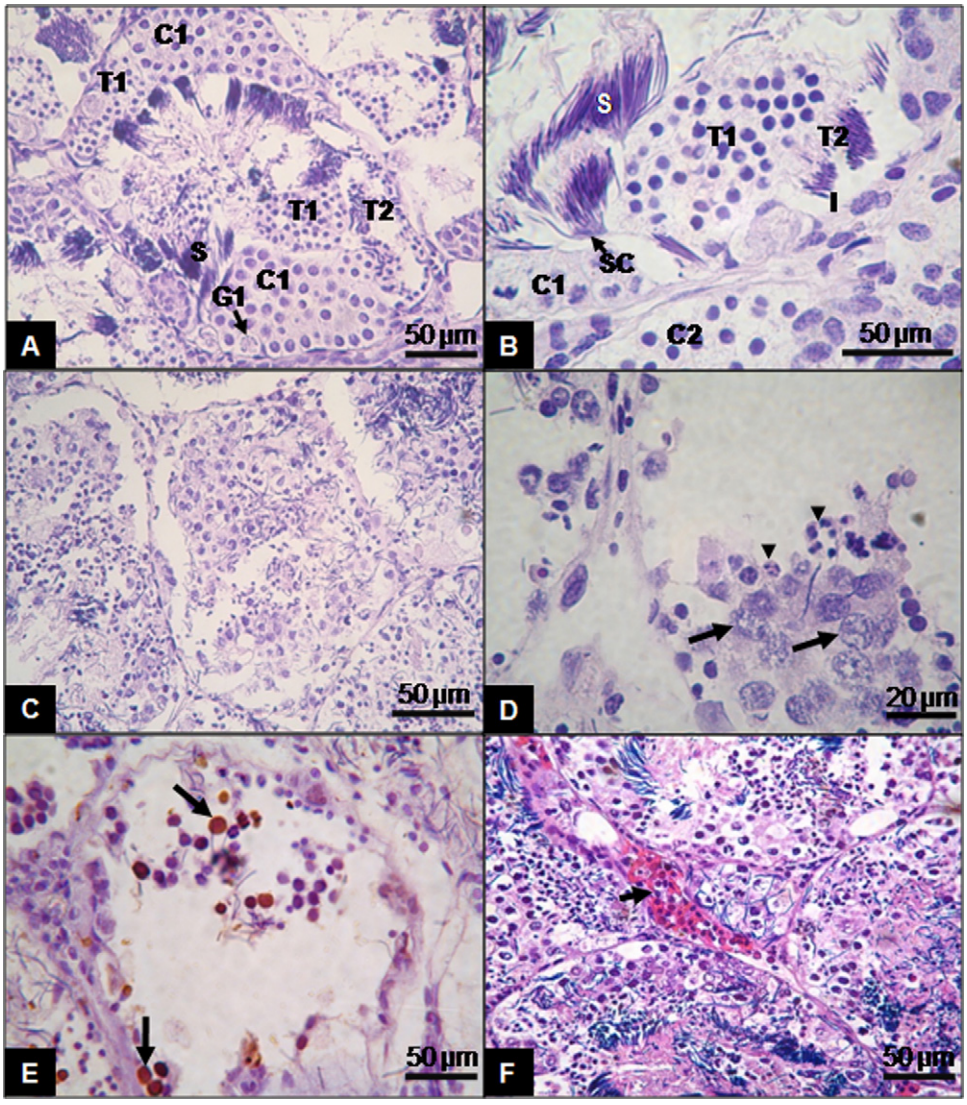

Fig. 5. Light micrographs of testes tissue of *Rhinella arenarum* after treatment with Cd. (A) Controls. Seminiferous tubules with preserved histoarchitecture. Note the germ cells organized in cysts. G1, primary spermatogonia; C1, primary spermatocyte; T1, primary spermatid; T2, secondary spermatid; S, sperm. H&E stain. (B) Treated with 0.5 mg/kg. Normal development of spermatogenic cysts and of interstitial tissue can be observed (I). C1, primary spermatocyte; C2, secondary spermatocyte; T1, primary spermatid; T2, secondary spermatid; S, sperm. SC, Sertoli cell. H&E stain. (C) Treated with 5 mg/kg. Complete desorganization of the seminiferous tubules. H&E stain. (D) Treated with 5 mg/kg. Germ line cells with hydropic tumefaction (arrows) and others with signs of apoptosis (arrowhead). H&E stain. (E) Treated with 5 mg/kg. Some TUNEL-positive cells (arrows) can be observed in the seminiferous tubules. (F) Treated with 5 mg/kg. Disorganization of cysts and dilation of vascular lumens in the interstitial tissue (arrow). Masson's trichrome stain.

would be relatively deficient in germ cells compared to kidney or liver cells as reported by Fowler (2009). Oxidative stress would make *R. arenarum* oocytes more sensitive to Cd exposure during the early stages of development. On the other hand, irreversible damage to the interstitial tissue that is replaced by fibrous tissue could be attributed to oxidative aggression. These deleterious effects seriously compromise oogenesis and oocyte nuclear maturation at the intoxication dose of 5 mg/kg at which ovaries exhibited a higher Cd concentration than the control. Our finding was consistent with those observed in *X. laevis* injected in the dorsal lymphatic sac with $CdCl_2$ every other day for 21 days (Lienesch et al., 2000).

With respect to the effect of Cd on male gonads, alterations in sperm quality parameters were detected. These alterations could be due to a decrease in the synthesis of testosterone as demonstrated by Ghosh et al. (1987) or to an increase in sperm reactive oxygen species production with associated sperm membrane damage (Benoff et al., 2009). Sperm motility involves sliding between pairs of doublet microtubules (A and B) in the axoneme, a process that is ATP dependent. It is important to note that the two doses used did not produce the same effects at the sperm motility level. Thus, at 0.5 mg/kg, we observed an increase in the *in situ* motility, while at 5 mg/kg there was a significant decrease in straight progressive motility, which is required by the fertilizing sperm. The effect of the xenobiotic on sperm motility could be due to the inhibition of microtubule sliding in the axoneme as demonstrated in bovine sperm (Kanous et al., 1993) or to mitochondrial deformation in the midpiece as reported in sea urchin sperm (Au et al., 2001b). Studies in common carp (Chyb et al., 2001), rainbow trout (Dietrich et al., 2010), sea urchin (Au et al., 2000, 2001a) and rat sperm (El-Demerdash et al., 2004; Xu et al., 2001) showed that Cd affects sperm motility. Accumulation of Cd in the testis of males of *R. arenarum* intoxicated with the 5 mg/kg Cd dose could be associated with the histopathological findings in the gonad. At the level of the interstice, the deterioration of the endothelium of small blood vessels would suggest the existence of circulatory disorders such as hyperemia, reduction in the blood flow or hypoxia. Cd also produces

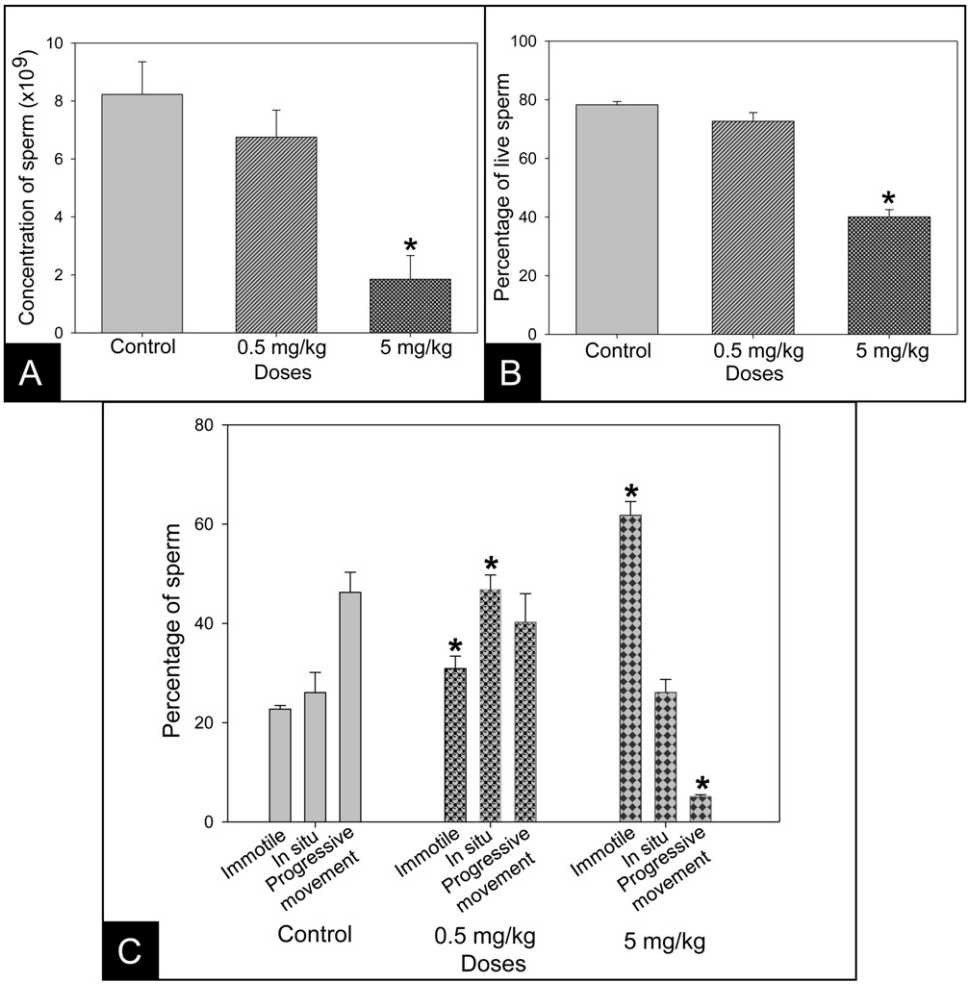

Fig. 6. Variation of (A) sperm concentration, (B) percentage of live sperm (unstained), (C) percentage of motile sperm (immotile sperm, *in situ* and progressive movement) of *R. arenarum* treated with different Cd doses. Data are reported as mean ± SE ($n = 5$ animals per dose). (*) indicates significant difference with respect to control ($p < 0.05$).

harmful effects at the level of the seminiferous epithelium due to hydropic tumefaction or focal necrosis observed in spermatogonia, spermatocytes and spermatids. The fact that Cd may compromise the process of spermatogenesis by damage to the spermatogonia is a predictor of permanent infertility. In agreement with the above, a decrease in secondary spermatogonial and primary spermatocytic stages in *R. hexadactyla Lesson* (Kasinathan et al., 1987) and *B. melanostictus* (Biswas et al., 1976) were observed.

In conclusion, on the basis of the results of the present work, it seems clear that Cd compromises the reproductive health of the amphibian *R. arenarum*, the germ cells of the male gonad being more sensitive than female germ cells.

Bearing in mind the seriousness of the worldwide increase in toxic residues such as heavy metals and the ease with which the metal may be introduced into the trophic chain, we propose the analysis of sperm quality parameters and of oocytes nuclear maturation as bioassay tests of cadmium pollution.

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